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The bile salt sodium taurocholate induces *Campylobacter jejuni* outer membrane vesicle production and increases OMV-associated proteolytic activity.

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**Running title:** Sodium taurocholate induces *Campylobacter jejuni* OMVs.
SUMMARY

*Campylobacter jejuni,* the leading cause of bacterial acute gastroenteritis worldwide, secretes an arsenal of virulence-associated proteins within outer membrane vesicles (OMVs). *C. jejuni* OMVs contain three serine proteases (HtrA, Cj0511 and Cj1365c) which cleave the intestinal epithelial cell (IEC) tight and adherens junction proteins occludin and E-cadherin, promoting enhanced *C. jejuni* adhesion to and invasion of IECs. *C. jejuni* OMVs also induce IECs innate immune responses. The bile salt sodium taurocholate (ST) is sensed as a host signal to coordinate the activation of virulence-associated genes in the enteric pathogen *Vibrio cholerae.*

In this study, the effect of ST on *C. jejuni* OMVs was investigated. Physiological concentrations of ST do not have an inhibitory effect on *C. jejuni* growth until the early stationary phase. Co-culture of *C. jejuni* with 0.1% or 0.2% (w/v) ST stimulates OMV production, increasing both lipid and protein concentrations. *C. jejuni* ST-OMVs possess increased proteolytic activity and exhibit a different protein profile compared to OMVs isolated in the absence of ST. ST-OMVs exhibit enhanced cytotoxicity and immunogenicity to T84 IECs and enhanced killing of *Galleria mellonella* larvae. ST increases the level of mRNA transcripts of the OMVs-associated serine protease genes and the *cdtABC* operon that encodes the cytolethal distending toxin. Co-culture with ST significantly enhances the OMVs-induced cleavage of E-cadherin and occludin. *C. jejuni* OMVs also cleave the major endoplasmic reticulum (ER) chaperone protein BiP/GRP78 and this activity is associated with the Cj1365c protease. This data suggests that *C. jejuni* responds to the presence of physiological concentrations of the bile salt ST which increases OMV production and the synthesis of virulence-associated factors that are secreted within the OMVs. We propose that these events contribute to pathogenesis.
INTRODUCTION


A defining feature of bacterial pathogens is the ability to sense host metabolites and precisely co-ordinate the expression of multiple virulence factors (Finlay *et al.*, 1997, Fang *et al.*, 2016a, Fang *et al.*, 2016b, Olive *et al.*, 2016). Bile is a well-characterised host metabolite, biosynthesised from cholesterol by hepatocytes in the liver (Joyce *et al.*, 2016, Vitek *et al.*, 2016). Bile is excreted from the gall bladder into the small intestine, particularly in the duodenum, jejunum and proximal ileum. The physiological concentration of bile in the intestine can be as high as 20 mM in the duodenum, then progressively decreasing to 10 mM in the jejunum, reaching as little as 4 mM in the ileum (Hofmann *et al.*, 2008, Maldonado-Valderrama *et al.*, 2011). In the intestine, bile emulsifies dietary fats and facilitates absorption of lipid nutrients. In addition, owing to the heterogeneous mixture of bile salts, bilirubin, phospholipids, cholesterol and enzymes, bile also has detergent-like toxicity and functions as a potent antimicrobial agent, solubilising membrane lipids including the dissociation of bacterial membrane proteins (Hofmann *et al.*, 1967, Begley *et al.*, 2005, Garidel *et al.*, 2007, Boyer, 2013). For many enteric pathogens including *Escherichia coli* O157:H7, *Vibrio cholerae, Shigella flexneri* and *Vibrio parahaemolyticus*, bile acts as a signal to modulate global gene expression of virulence factors (Alam *et al.*, 2010, Gotoh *et al.*, 2010, Faherty *et
The conjugated bile salt sodium taurocholate (ST) has been shown to act as a host signal for the *V. cholerae* 7th pandemic O1 El Tor biotype strain N16961 (Yang et al., 2013), inducing intermolecular di-sulphide bond formation in the transmembrane transcription activator TcpP (Yang et al., 2013). More recently, the mechanisms by which *V. parahaemolyticus* senses bile salts via the type III secretion system 2 (T3SS2) regulators VtrA and VtrC have been identified (Li et al., 2016). Analysis of the crystal structure of the periplasmic domains of the VtrA/VtrC heterodimer revealed that VtrA and VtrC form a protein complex on the surface of the outer membrane creating a barrel-like structure that binds to bile salts and induces *V. parahaemolyticus* to secrete toxins (Li et al., 2016).

As with the other *Proteobacteria* pathogens *Haemophilus ducreyi*, *Aggregatibacter actinomycetemcomitans* and *Shigella dysenteriae*, *C. jejuni* possesses CDT which interferes with normal cell cycle progression, resulting in G2 arrest (Pickett et al., 1996, Whitehouse et al., 1998, Purdy et al., 2000). CDT (encoded by the *cdtABC* operon) is the only identified toxin in *C. jejuni*, yet the role that host metabolites such as bile salts play in inducing CDT secretion is unclear. Like many other enteric pathogens, *C. jejuni* encounters bile salts during human infection (Drion et al., 1988, Raphael et al., 2005). The bile salt sodium deoxycholate induces differential expression of *C. jejuni* virulence genes, notably increasing the expression of *Campylobacter* Invasion Antigen (Cia) genes, leading to enhanced invasion of IECs by *C. jejuni* (Malik-Kale et al., 2008). However the effects of the taurocholate-conjugated hydrophilic bile salts such as ST on *C. jejuni* physiology or the role of ST as a host cellular signal for *C. jejuni* have not been previously investigated.

Outer membrane vesicles (OMVs) are nano-sized structures (50–250nm in diameter) that are released by all Gram-negative bacteria (Kulkarni et al., 2014, Schwechheimer et al., 2015). OMVs are enriched with an active molecular cargo of virulence factors such as adhesins,
invasins, toxins, proteases, antigenic proteins and non-protein antigens such as lipo-polysaccharide (LPS). OMVs are associated with a multi-faceted role in bacterial pathogenesis (Schertzer et al., 2013, Kaparakis-Liaskos et al., 2015, Schwechheimer et al., 2015, Vanaja et al., 2016). So far only limited studies on the role of stress response pathways on bacteria vesiculation have been reported (McBroom et al., 2007, Macdonald et al., 2013, Schwechheimer et al., 2013a, Schwechheimer et al., 2013b, Schwechheimer et al., 2014). Whilst these studies have contributed to the understanding of the role of OMVs in bacterial pathophysiology, there are probably many roles of OMVs in bacterial pathogenesis that are still poorly defined. These questions are particularly relevant for understanding C. jejuni pathogenesis that in comparison to other enteric pathogens, is poorly understood.

C. jejuni produces OMVs enriched with an active arsenal of virulence factors (Lindmark et al., 2009, Elmi et al., 2012). More recently we have highlighted the role of three serine proteases (HtrA, Cj0511 and Cj1365c) secreted in C. jejuni OMVs which cleave the intestinal epithelial cell (IEC) tight and adherens junction proteins occludin and E-cadherin, promoting enhanced C. jejuni adhesion to and invasion of IECs (Elmi et al., 2016). The role of C. jejuni OMVs in pathogenesis and the emerging role of bile salts such as ST in inducing bacterial virulence led to the investigation of the effect of ST on C. jejuni OMVs production. ST stimulates C. jejuni 11168H OMVs production, increasing both the protein concentration and lipid content of OMVs. ST enhances the proteolytic activity associated with C. jejuni OMVs, leading to enhanced OMVs cytotoxicity and immunogenicity to IECs. ST increases the level of mRNA transcripts of the OMVs-associated serine protease genes and also the cdtABC operon. Collectively, our data demonstrates that C. jejuni responds to physiological concentrations of the bile salt ST, increasing OMVs production and the synthesis of virulence-associated factors that are secreted within the OMVs.
RESULTS

Co-culture with sodium taurocholate increases both the protein concentration and lipid content of C. jejuni OMVs

As bile salts have bactericidal properties (Begley et al., 2005, Merritt et al., 2009), the effect of ST on the growth of C. jejuni was investigated. No significant difference in growth rate as measured by OD₆₀₀ readings (Figure 1A) or CFU counts (Figure 1B) was observed when C. jejuni 11168H was cultured in Brucella broth in the presence of 0.1% or 0.2% (w/v) ST compared to bacteria cultured in Brucella broth alone. To investigate whether ST had any damaging effects on C. jejuni membranes, LIVE/DEAD BacLight staining was performed using microplate fluorescence and confocal microscopic analysis of C. jejuni 11168H after co-culture in Brucella broth for 12 hours in the presence of 0.1% or 0.2% (w/v) ST. No reduction in the numbers of live C. jejuni cells was observed in the presence of ST (Figure 1C), indicating that the viability of C. jejuni cells were not affected by either of these concentrations of ST. Confocal microscopic analysis of C. jejuni 11168H also revealed the membrane integrity of the bacterial cells was not affected (Figure 1D).

OMVs isolated from C. jejuni 11168H cultured in the presence of 0.1% or 0.2% (w/v) ST (ST-OMVs) had significantly higher protein concentrations than OMVs isolated from the same volume of C. jejuni 11168H cultured in the absence of ST (Figure 2A). In addition, the lipid content of C. jejuni ST-OMVs was also increased compared to OMVs isolated from C. jejuni 11168H cultured in the absence of ST (Figure 2B). To establish the role of ST in enhancing C. jejuni OMV formation, the amount of Keto-deoxy-d-manno-8-octanoic acid (Kdo) associated with OMVs was assessed. Kdo is a characteristic constituent of Gram-negative bacterial LPS and LOS (Brade et al., 1984). The amount of Kdo associated with OMVs isolated from C. jejuni 11168H cultured in the presence of 0.1% or 0.2% (w/v) ST was significantly increased (Figure 2C). To extend these findings, OMVs were separated on SDS-PAGE gel and stained
with silver stain to visualise LOS. The presence of 0.1% or 0.2% (w/v) ST resulted in enhanced staining of OMVs-associated LOS compared OMVs isolated in the absence of ST (Figure 2D).

Proteomic analysis indicates an increase in the number of proteins associated with C. jejuni ST-OMVs

To further investigate the significance of ST on C. jejuni OMV production, the proteome of OMVs isolated from C. jejuni 11168H grown in the presence of 0.2% (w/v) ST was investigated. A total of 185 proteins were identified (Table S1), 131 of which were also identified in our earlier proteomic analysis of C. jejuni 11168H OMVs (Elmi et al., 2012). The proportions of proteins from each cluster of orthologous groups (COG) categorization are shown in Figure 3. Of the 34 new proteins identified as specific to ST-OMVs, one of the Campylobacter invasion antigens CiaI (Cj1450) (Buelow et al., 2011) was identified as well as the Cj1365c protease and the bile salt response protein Cj0561c. Cj1614 and Cj0755, which are involved in the binding, uptake or export of trace metals, and the 2-oxoglutarate-acceptor oxidoreductase subunit OorABC were also identified along with Cj0628, a putative autotransporter protein that functions as an adhesin with a role in colonisation (Ashgar et al., 2007).

Co-culture with sodium taurocholate significantly enhances OMVs proteolytic activity

C. jejuni 11168H OMVs contain biologically active serine proteases that interact with IEC proteins to enhance C. jejuni adherence to and invasion of IECs (Elmi et al., 2012, Elmi et al., 2016). C. jejuni 11168H ST-OMVs exhibited a statistically significant enhanced proteolytic activity compared to OMVs isolated from C. jejuni 11168H cultured in the absence of ST (Figure 4).
**ST-OMVs exhibit enhanced cytotoxicity and immunogenicity to T84 intestinal epithelial cells**

Given ST-OMVs exhibit enhanced proteolytic activity, the cytotoxicity of *C. jejuni* OMVs and ST-OMVs co-incubated with T84 IECs for 24 hours was investigated. Increased levels of cytosolic lactate dehydrogenase (LDH) after co-incubation of T84 IECs with ST-OMVs was observed compared to co-incubation with OMVs (Figure 5A). The induction of interleukin-8 (IL-8) from T84 cells by OMVs and ST-OMVs was quantified using ELISA (enzyme-linked immunosorbent assay). ST-OMVs significantly enhanced induction of IL-8 compared to co-incubation with OMVs (Figure 5B). The effect of ST on the OMV-induced up-regulation of IL-8 was also investigated. ST enhanced the mRNA transcription of IL-8 in T84 IECs (Figure 5C).

**ST-OMVs exhibit enhanced cytotoxicity in the *Galleria mellonella* model of infection**

To investigate the effect of ST on *C. jejuni* OMVs in vivo, 11168H ST-OMVs and OMVs were injected into *G. mellonella* larvae. Injection with ST-OMVs resulted in an increase in killing of larvae compared to injection with OMVs (Figure 6). To investigate whether ST had any toxicity towards *G. mellonella* larvae, ST alone was also injected. No killing was observed in the *G. mellonella* larvae injected with 10 μl of either 0.1% or 0.2% (w/v) ST.

**Co-culture with sodium taurocholate results in differential expression of genes encoding OMV-associated CDT and serine proteases**

The effects of ST on the relative expression of genes involved in *C. jejuni* cytotoxicity (*cdtABC* operon) and proteolytic activity (*htrA, Cj0511* and *Cj1365c*) were investigated. Total RNA was isolated from *C. jejuni* 11168H cultured in the absence of ST or in the presence of 0.1% or 0.2% (w/v) ST. Expression of both the *cdtABC* operon (Figure 7A) and the serine protease
genes (Figure 7B) was significantly enhanced relative to the level of the housekeeping DNA gyrase gene *gyrA* (Ritz *et al.*, 2009). In agreement with the proteolytic and cytotoxicity data, these data imply that ST enhances the expression of *C. jejuni* 11168H virulence-associated genes to facilitate enhanced proteolytic activity, cytotoxicity and immunogenicity.

**Co-culture with sodium taurocholate significantly enhances the OMV-induced cleavage of occludin and E-cadherin in vitro**

*C. jejuni* OMVs cleave the major tight junction (TJ) and adheren junctions (AJ) proteins occludin and E-cadherin (Elmi *et al.*, 2016). Recombinant occludin and E-cadherin were co-incubated with OMVs and ST-OMVs. Incubation with ST-OMVs resulted in an increase in the cleaved form of both occludin and E-cadherin compared to incubation with OMVs (Figure 8AB).

**C. jejuni 11168H OMVs cleave the major endoplasmic reticulum (ER) chaperone protein BiP/GRP78**

The Cj1365c protease possesses a subtilase domain. The amino acid sequences of the subtilase domain share 20% identity with the A subunit (SubA) of the subtilase cytotoxin SubAB of Shiga toxigenic *Escherichia coli* (STEC). STEC SubA mediates an unusual toxicity of the SubAB5 toxin with an extreme substrate specificity towards ER chaperone GRP78/BiP (Paton *et al.*, 2006). Despite the low level of sequence identity, the position of the key residues in the catalytic serine active site of Cj1365c at position 274 (numbering corresponds to the protein without signal sequence) is conserved in STEC SubA, suggesting that GRP78/BiP may serve as a substrate for the Cj1365c protease. The activity of OMVs isolated from *C. jejuni* in the presence or absence of ST to cleave recombinant GRP78/BiP was therefore investigated. *C. jejuni* OMVs were able cleave GRP78/BiP and this cleavage was enhanced with ST-OMVs.
OMVs isolated from the 11168H wild-type strain and from the three protease mutants (htrA, Cj0511 and Cj1365c) were co-incubated with recombinant GRP78/BiP. Only OMVs isolated from the Cj1365c mutant exhibited reduced ability to cleave GRP78/BiP (Figure 9B).
DISCUSSION

*C. jejuni* OMVs are cytotoxic, immunogenic and proteolytic (Elmi *et al.*, 2016). However the effect of host metabolites such as bile salts on *C. jejuni* OMVs secretion, content and function has not been studied. Recently genetic, biochemical and transcriptome analyses have been used to reveal the role of bile salts in inducing the pathogenesis of different enteric bacteria (Joyce *et al.*, 2016, Sistrunk *et al.*, 2016). Bile salts mediate reciprocal host-pathogen crosstalk (Gotoh *et al.*, 2010, Chaand *et al.*, 2013) and there is increasing evidence supporting the role of bile salts in regulating bacterial virulence gene expression (Yang *et al.*, 2013, Li *et al.*, 2016, Xue *et al.*, 2016). ST plays an important role in inducing *V. cholerae* virulence gene expression (Xue *et al.*, 2016), However the effect of ST on *C. jejuni* has not been investigated. In this study, we have characterised the role of ST in modulating *C. jejuni* 11168H OMVs secretion, content and function. Both the protein and lipid concentration of 11168H OMVs were increased when *C. jejuni* was cultured in the presence of physiologically relevant concentrations of ST (0.1% and 0.2% w/v ST). Changes in OMV secretion, content and function under various growth conditions have also been observed in a number of other bacterial pathogens (Tashiro *et al.*, 2010, Choi *et al.*, 2014, Metruccio *et al.*, 2016).

Proteomic analysis of *C. jejuni* 11168H ST-OMVs identified all 131 proteins previously identified associated with 11168H OMVs (Elmi *et al.*, 2012), but also a further 34 proteins, including proteins expected to be involved *C. jejuni* response to bile salts such as Cj0561c for efficient colonisation (Guo *et al.*, 2008). The presence of CiaI (Cj1450) was of note as this is the first time that one of the Cia proteins has been identified associated with OMVs. Previously the secretion mechanism of CiaI via the flagellum was shown to be independent of host cell contact (Barrero-Tobon *et al.*, 2014). During colonisation of the human intestine, *C. jejuni* will be exposed to a number of different bile salts. The bile salt sodium deoxycholate (SD) increases the expression of *cia* genes (Malik-Kale *et al.*, 2008), however the effect of ST on *cia* gene
expression has not been investigated. It is possible that a combination of SD and ST would increase the number of Cia proteins that could be identified associated with *C. jejuni* OMVs. Secretion of Cia proteins within OMVs would provide a new mechanism for the delivery of these virulence determinants into host cells.

The three proteases (HtrA, Cj0511 or Cj1365c) associated with OMVs are conserved in *C. jejuni* and inactivation of each has been linked to the attenuation of *C. jejuni* physiology, virulence and/or immunogenicity (Brondsted *et al.*, 2005, Karlyshev *et al.*, 2014, Jowiya *et al.*, 2015). The enhanced proteolytic activity of 11168H ST-OMVs indirectly indicates a more pathogenic *C. jejuni* response during colonisation of a host after the bacteria senses bile salts. The cytotoxic and immunogenic properties of OMVs are well characterised (Kaparakis-Liaskos *et al.*, 2015, Pathirana *et al.*, 2016). The enhanced cytotoxicity and immunogenicity of ST-OMVs may be associated with the increase in concentrations of proteases and CDT secreted in ST-OMVs, based on the qPCR analysis which showed that ST significantly up-regulates expression of *htrA*, *Cj0511*, *Cj1365* and the *cdtABC* operon. This supports an emerging consensus that bile salts in the intestine play important roles in modulating bacterial gene expression. The enhanced expression of *cdtA* is consistent with the dynamics of operon expression with a recent study reporting that there is a linear relationship between transcription distance and gene expression in operons (Lim *et al.*, 2011). This observation also indicates the importance of CdtA and CdtC which are involved in delivery of CdtB which possesses type I deoxyribonuclease-like activity that mediates IECs DNA damage, triggering the response of the cell cycle checkpoint and results in G2 arrest (Lara-Tejero *et al.*, 2001). Internalisation of CdtB is also positively correlated with CdtA and CdtC as purified CdtB alone has no effect on IECs, but when combined with CdtA and CdtC, IECs exhibited cell cycle arrest in the G2/M phase (Elwell *et al.*, 2001, Lara-Tejero *et al.*, 2001). However in contrast to *Vibrio* species, the mechanism(s) of ST interactions with *C. jejuni* remains unclear. *C. jejuni* lacks orthologues of
*V. parahaemolyticus* VtrA and VtrC that create a barrel-like structure that can bind to bile salts and trigger the production of cholera toxin. ST might cause changes in *C. jejuni* outer membrane proteins or alter levels and/or activities of yet unidentified *C. jejuni* proteins that could regulate synthesis and protein folding machinery or post-translational modifications. *C. jejuni* has orthologues of the Maintenance of lipid asymmetry (Mla) pathway proteins which retrograde phospholipid transport from the OM back to the IM and are involved in bacterial OMVs biogenesis in *V. cholerae* and *Haemophilus influenzae* (Roier et al., 2016a, Roier et al., 2016b). However further studies are required to identify the precise mechanism of *C. jejuni* ST-induced OMV biosynthesis.

ST-OMVs enhanced the cleavage of both occludin and E-cadherin and this was more pronounced for occludin than for E-cadherin. One explanation is that *C. jejuni* may preferentially bind and cleave apically located tight junction proteins such as occludin that function to seal the paracellular passage that is crucial for IEC integrity. *C. jejuni* also invades IECs via an actin filament-mediated mechanism and actin filaments interface with tight junction proteins (Biswas et al., 2003). In addition, this result reinforces previous findings that correlated significant decrease in transepithelial electrical resistance (TEER) with redistribution and dephosphorylation of occludin instead of E-cadherin (Chen et al., 2006). Loss of occludin in IECs infected with *C. jejuni* also led to significant alteration in tight junction transmembrane proteins (MacCallum et al., 2005). Similar studies in *Escherichia coli* (EPEC) and *Salmonella enterica* serovar *Typhimurium* also showed a pathogenic mechanism associated with occludin-specific redistribution (Sakakibara et al., 1997, McNamara et al., 2001, Bertelsen et al., 2004). It appears that a number of virulence-associated serine proteases secreted by different enteric pathogens preferentially alter the expression and distribution of occludin, indicating that occludin might be an important IEC target that shapes the bacterial-host interplay (Guttman et al., 2008, Awad et al., 2017, Eichner et al., 2017). Future studies
should focus on understanding the precise mechanisms of OMV-associated serine proteases that enteric pathogens including *C. jejuni* use to interact with and cleave/modify occludin.

The ER chaperone GRP78/BiP protein is highly conserved and essential for the survival of eukaryotic cells. GRP78/BiP also maintains the permeability barrier of the ER membrane and plays a crucial role in the unfolded-protein response (UPR) as the ER stress-signalling master regulator. For the first time *C. jejuni* OMVs have also been shown to cleave the recombinant ER chaperone GRP78/BiP, a key cellular target for AB$_5$ toxins (Paton *et al.*, 2006, Beddoe *et al.*, 2010, Paton *et al.*, 2010). This activity was only reduced in OMVs isolated from a *Cj1365c* mutant, suggesting that it is this protease responsible for the cleavage of GRP78/BiP. *Cj1365c* is a multi-domain protein, containing an autotransporter domain as well as a subtilisin-like serine domain. Serine proteases belonging to the family of subtilisin-like proteases are secreted by a wide variety of bacterial pathogens and have been shown to cleave host proteins including immunoglobulins, complement compounds and proteins of the extracellular matrix (Male, 1979, Juarez *et al.*, 1999, Mortensen *et al.*, 2011). Considering previous studies have suggested that the expression of CDT is not directly linked to *C. jejuni* disease severity (Abuoun *et al.*, 2005, Mortensen *et al.*, 2011), this observation may also highlight the importance of the *Cj1365c* protease in *C. jejuni* pathogenesis. The *Cj1365c* protease has previously been suggested to be one of the *C. jejuni* virulence factors associated with the development of bloody diarrhoea, independent of pVir plasmid (Louwen *et al.*, 2006). The novel AB$_5$ toxin that cleaves GRP78/BiP also occurs in the loss of enterocyte effacement negative STEC O113:H21 outbreak strain (Newton *et al.*, 2009).

In summary, ST is important not only for the up-regulation of the genes encoding HtrA, Cj0511, *Cj1365c* and CDT *in vitro*, but also for the modulation of *C. jejuni* OMV secretion, content and function. Our findings and other recent reports also highlight the importance of ST in inducing bacterial virulence. Enhanced *C. jejuni* vesiculisation induced by ST can also be
linked to enhanced cytotoxicity to both IECs and *Galleria mellonella* larvae as well as enhanced immunogenicity and proteolytic activity. This data suggests that *C. jejuni* responds to the presence of the bile salt ST by increasing OMV production and changing the protein content of OMVs to enhance pathogenesis.

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EXPERIMENTAL PROCEDURES

**Bacterial strains and culture conditions**

The *C. jejuni* wild-type strain used in this study was 11168H, a hypermotile derivative of the sequence strain NCTC11168 that shows higher levels of caecal colonisation in a chick colonisation model (Jones *et al.*, 2004). *C. jejuni* strains were grown either on blood agar plates containing Columbia agar base (Oxoid, UK) supplemented with 7% (v/v) horse blood (TCS Microbiology, UK) and *Campylobacter* Selective Supplement (Oxoid) or in Brucella broth (Oxoid) with shaking at 75 rpm in a microaerobic chamber (Don Whitley Scientific, UK) containing 85% N₂, 10% CO₂ and 5% O₂ at 37°C. Kanamycin (Sigma-Aldrich, UK) was added to blood agar plates or to Brucella broth as required at the concentration of 50 μg/ml. Unless otherwise stated, *C. jejuni* strains were grown on blood agar plates for 24 h prior to use in all subsequent experiments.

**Isolation and Quantification of *C. jejuni* OMVs**

*C. jejuni* OMVs were isolated as described previously (Elmi *et al.*, 2012). *C. jejuni* from a 24 h blood agar plate were resuspended in 1 ml Brucella broth and used to inoculate 100 ml pre-equilibrated Brucella broth to an OD₆₀₀ of 0.1. Cultures were grown for 12 h (OD₆₀₀ ≈ 1.2) then centrifuged at 4,000 x g for 30 mins at 4°C. The resulting supernatant was filtered through a 0.22 μm membrane (Millipore, UK) then the filtrate concentrated to 2 ml using an Ultra-4 Centrifugal Filter Unit with a nominal 10 kDa cutoff (Millipore). The concentrated filtrate was ultra-centrifuged at 150,000 x g for 3 h at 4°C using a TLA 100.4 rotor (Beckman Instruments, USA). All isolation steps were performed at 4°C and the resulting OMVs pellet was resuspended in phosphate buffered saline (PBS) and stored at -20°C. OMVs samples were plated out on blood agar plates and incubated under both microaerobic and aerobic conditions for 72 h to confirm the absence of viable bacteria. The protein concentration of the OMVs was
quantified using a bicinchoninic-acid assay (BCA Protein Assay; Thermo Fisher Scientific, UK) using BSA as the protein standard. 10 μg of each OMVs sample, based on protein concentration, was analysed using SDS-PAGE and stained using Silver stain Reagent (Thermo Fisher Scientific). The LOS profiles of the OMVs were examined using standard methods (Naito et al., 2010). The OMVs samples were separated on 12% (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then visualised using silver staining.

**Further quantification of OMVs**

Further OMV quantification was performed as described previously (Lee et al., 1999) by measuring keto-deoxy-d-manno-8-octanoic acid (Kdo) content. Keto-deoxy-d-manno-8-octanoic acid from *E. coli* LPS (Sigma-Aldrich) was used as the standard. For the quantification of the lipid content of OMVs, the fluorescent lipophilic dye FM4-64 (Thermo Fisher Scientific) was used to a final concentration of 5 μg/ml (McBroom et al., 2006). OMVs alone and the FM4-64 probe alone were used as negative controls.

**Mass spectrometry analysis**

Proteins from three separate *C. jejuni* OMVs preparations were resolved on SDS-PAGE gels then submitted to the Moredun Proteomics Facility for analysis. Each gel lane was excised and sliced horizontally from top to bottom to yield equal gel slices 2.5 mm deep. Each of the resulting gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinosolysis procedures (Shevchenko et al., 1996). Digests were transferred to low-protein-binding HPLC sample vials immediately prior to liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) analysis. Liquid chromatography was performed using a Dionex Ultimate 3000 nano-HPLC system (Thermo Fisher Scientific) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column
compartment, a UVD-3000 UV detector, a LPG-3600 dual-gradient micropump and a SRD-3600 solvent rack controlled by Chromeleon™ chromatography software (www.thermoscientific.com/dionex). A micro-pump flow rate of 246 µl/min was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of 3 µl/min through a 5 cm x 200 µm ID monolithic reversed phase column (Thermo Fisher Scientific) maintained at 50°C. Samples of 4 µl were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8-45% solvent B (80% acetonitrile (Rathburn Chemicals, UK), 0.1% v/v formic acid) and directed through a 3 nl UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (amaZon-ETD, Bruker Daltonics, Germany) via a low-volume (50 µl/min maximum) stainless steel nebuliser (Agilent Technologies, UK / G1946-20260) and ESI. Parameters for tandem MS analysis were based on those described previously (Batycka et al., 2006).

**Database mining**

Deconvoluted MS/MS data in mgf (Mascot Generic Format) format was imported into ProteinScape™ V3.1 (Bruker Daltonics) proteomics data analysis software for downstream mining of databases utilising the Mascot™ V2.4.1 (Matrix Science, UK) search algorithm. The protein content of each individual gel slice was established using the “Protein Search” feature of ProteinScape™, whilst separate compilations of the proteins contained in all gel slices for each sample were produced using the “Protein Extractor” feature of the software. Mascot search parameters were set in accordance with published guidelines (Taylor et al., 2005) using fixed (carbamidomethyl “C”) and variable (oxidation “M” and deamidation “N,Q”) modifications along with peptide (MS) and secondary fragmentation (MS/MS) tolerance values of 0.5 Da and allowing for a single $^{13}$C isotope. Molecular weight search (MOWSE) scores
attained for individual protein identifications were inspected manually and considered significant only if a) two peptides were matched for each protein, with each matched peptide containing an unbroken “b” or “y” ion series represented by of a minimum of four contiguous amino acid residues or b) one peptide was matched for each protein, containing an unbroken “b” or “y” ion series represented by of a minimum of eight contiguous amino acid residues.

Files were searched with MASCOT software against C. jejuni NCTC 11168 protein databases derived from genomic sequence available at NCBI (Genbank, AL111168) (http://www.ncbi.nlm.nih.gov/).

The NCTC 11168 annotated genome sequence indicates Clusters of Orthologous Group (COG) assignments (Tatusov et al., 1997) which were used to predict functional classification. Additionally the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search) and The Koyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) (Kanehisa et al., 2000) were used to investigate protein function.

**Quantitative determination of OMVs proteolytic activity**

The proteolytic activity of OMVs was determined using a Protease Fluorescent Detection Kit (Sigma-Aldrich) using a FITC-labelled casein substrate as described previously (Elmi et al., 2016). OMVs (10 µg in 10 µl, based on protein concentration) from C. jejuni cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST were mixed with 20 µl incubation buffer and 20 µl substrate then incubated at 37°C in the dark for 24 h. The reaction was stopped by adding 200 µl 0.6 N trichloroacetic acid to precipitate any remaining substrate, which was removed by centrifugation at 10,000 x g for 10 mins at 4°C. The supernatant obtained (10 µl) was diluted in 1 ml assay buffer. The digested FITC-casein substrate has absorption / emission maxima at 485 nm / 535 nm, and fluorescence intensity was recorded.
using a Multi-Mode Microplate reader (Molecular Devices, UK). Bovine trypsin (1 µg/ml) was used as positive control in all experiments.

**Cell lines, media and culture conditions**

The human T84 colon cancer epithelial cells were obtained from the National Type Culture Collection. T84 cells were maintained at sub-confluence in DMEM / F-12 (Invitrogen, UK) supplemented with 10% (v/v) FCS, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich) at 37°C in a 5% CO₂ humidified atmosphere. Cells were split around 80-90% confluence and seeded at 5 x 10⁶ cells per well into 24-well tissue culture plates (Corning Glass Works, Netherlands) using 1 ml volumes of cell culture media per well. Medium was replenished every 2 days. For ELISA experiments, the medium was removed and monolayers washed three times with PBS, then maintained in antibiotic-free medium supplemented with 10% (v/v) serum (Invitrogen) for 24 h before each experiment.

**Cytotoxicity detection assay**

The CytoTox 96® non-radioactive cytotoxicity assay (Promega, UK) was used to quantify the cell damage induced by co-culture T84 cells with OMVs isolated from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST. Briefly, T84 cells were challenged with 100 µg OMVs based on protein concentration. After co-incubation at 37°C for 24 h, cell supernatants were analysed for the release of lactate dehydrogenase (LDH). Non-challenged cells represented the 0% cytotoxicity negative control. Total lysis of cells following treatment with 1% (v/v) Triton X-100 represented the 100% cytotoxicity positive control.
Enzyme-linked immunosorbent assay (ELISA) for IL-8 quantitation

T84 cells were co-cultured with 100 µg or 10 µg of OMVs based on protein concentration isolated from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST for 24 h. The levels of IL-8 secretion were assessed using a commercially available sandwich ELISA kit according to manufacturer’s instructions (E-Biosciences, UK). Detection was performed using a Dynex MRX II 96 well plate reader (Dynex, U.S.A) at an absorbance of 450 nm ($A_{450}$) and analysed using Revelation software (Dynex).

Galleria mellonella larvae model of infection

*G. mellonella* larvae (LiveFoods Direct, UK) were kept on wood chips at 16°C. Experiments were performed with slight modifications from the original published methodology (Champion *et al.*, 2010) as described previously (Gundogdu *et al.*, 2011). Briefly, 5 µg of OMVs based on protein concentration isolated from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST, in a 10 µl volume were injected into the right foremost leg of the *G. mellonella* larvae by micro-injection (Hamilton, Switzerland). For each experiment, 10 *G. mellonella* larvae were injected and experiments were repeated three times using larvae of the same approximate weight. Controls were both non-injected larvae or larvae injected with 10 µl of sterile PBS. Larvae were incubated at 37°C and survival recorded at 24 h intervals for 72 h.

Quantitative real-time polymerase chain reaction (qRT-PCR).

For measurements of gene expression, total RNA from either *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST or T84 cells was extracted with Qiagen RNAeasy mini kit (Life Technologies). DNA contamination was removed with DNA-free treatment (Ambion). The purified RNA was quantified using a Nanodrop machine.
(NanoDrop Technologies, UK). Complementary DNA (cDNA) was synthesised with a Superscript III first-strand synthesis system (Life Technologies) according to the manufacturer's protocol. Briefly, 2 µg total RNA was used for the reverse-transcription reaction mixture (Fisher Scientific) using either random hexamers or oligo(dT) primers. Quantitative real-time PCR reactions were performed in triplicate using SYBR green master mix (Applied Biosystems) with ABI7500 machine (Applied Biosystems). Relative gene expression comparisons were performed using ΔΔCT method (Schmittgen et al., 2008), (where CT is threshold cycle) normalising the mean cycle threshold of each gene to the gyrA gene, which is considered a stably expressed housekeeping gene (Ritz et al., 2009). Primer efficiencies were tested with genomic DNA dilution series and primer sequences are listed in Table 1.

**In vitro cleavage of occludin and E-cadherin by C. jejuni OMVs**

The cleavage of recombinant TJ or AJ proteins (occludin or E-cadherin respectively) by OMVs was determined as described previously (Baek et al., 2011, Elmi et al., 2016). Briefly, OMVs (10 µg based on protein concentration) from C. jejuni cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST were incubated with 1 µg recombinant human occludin or E-cadherin (R&D Systems, UK) in PBS at 37°C for 16 h. For analysis of occludin or E-cadherin cleavage, reactions were mixed with 2X sample loading buffer (125 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) 14.3 M β-mercaptoethanol, 10% (v/v) glycerol, 0.006% (w/v) bromophenol blue) and boiled for 10 min. Proteins were separated using 12% (w/v) Bis/Tris precast gels (Invitrogen) then transferred to nitrocellulose using an iBlot gel transfer device (Life Technologies). Membranes were incubated in a blocking buffer (2% (w/v) skimmed milk (Tesco, UK) in PBS) for 1 h at room temperature. After removal of blocking buffer, membranes were rinsed three times with 0.1% (v/v) Tween-20 in PBS then incubated 1 h at room temperature with primary rabbit anti-occludin or primary mouse anti-E-Cadherin
or (Abcam, UK) (1:1,000). Following primary antibody incubation, membranes were washed four times with 0.1% (v/v) Tween-20 in PBS followed by incubation with an infrared fluorescence-conjugated secondary antibody (either goat anti-mouse IR800 or goat anti-rabbit IR680 (Licor Biosciences, UK) prepared in a 1:10,000 dilution of blocking buffer) at room temperature for 1 h. Membranes were scanned and analysed using a Licor Odyssey® (Licor Biosciences).

**in vitro cleavage of GRP78/BiP**

*C. jejuni* OMVs cleavage of GRP78/BiP was performed as previously described (Nagasawa et al., 2014). Briefly, 20 µg of OMVs based on protein concentration were incubated with PBS buffer with GRP78/BiP (Fisher Scientific) in a final volume of 25 µl at 37°C for 16 h. The reactions were stopped by addition of 2X sample loading buffer. The samples were analysed by SDS-PAGE and western blot as described above using GRP78/BiP primary antibody.

**Statistical analysis**

All experiments represent at least three biological replicates with each experiment performed in triplicate. All data were analysed using Prism statistical software (Version 6, GraphPad Software, USA). Values were expressed as mean ± SEM. Variables were compared for significance using Two-Way Analysis of Variance (ANOVA) and the Bonferroni test with one asterisk (*) indicating a $p$ value between 0.01 and 0.05, two asterisks (**) indicating a $p$ value between 0.001 and 0.01 and three asterisks (***) indicating a $p$ value < 0.001.
### TABLES

Table 1. qRT-PCR primers used in this study.

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FIGURE LEGENDS

Figure 1. ST does not affect the growth or outer membrane integrity of *C. jejuni* 11168H wild-type strain.

Growth curves of *C. jejuni* 11168H cultured in Brucella broth without ST or Brucella broth supplemented with 0.1% (w/v) ST or 0.2% ST (w/v) quantified by (A) OD$_{600}$ or (B) Colony Forming Units (CFU). (C) Quantitative analysis of *C. jejuni* 11168H stained with LIVE/DEAD BacLight for the purpose of evaluating outer membrane integrity phenotype. (D) Representative fluorescence confocal images showing the relative live/dead cells of *C. jejuni* 11168H cultured without ST or with 0.1% (w/v) or 0.2% (w/v) ST. Cells were stained with LIVE/DEAD BacLight (Life Technologies) (green = viable cells, red = dead cells). Scale bar, 10 μm.

Figure 2. ST increases the protein concentration of OMVs from *C. jejuni* 11168H wild-type strain.

Quantification of the increase in OMVs isolated from *C. jejuni* 11168H in Brucella broth supplemented with 0.1% (w/v) or 0.2% (w/v) ST compared to without ST. The OD$_{600}$ of ST treated 11168H cultures were normalised to the OD$_{600}$ of untreated 11168H cultures for each OMV isolation. (A) BCA assay to determine protein concentration of OMVs. *, $P < 0.05$; ****, $P < 0.0001$. (B) Relative fold increase of OMVs determined using FM4-64 dye. *, $P < 0.05$; ****, $P < 0.0001$. (C) Relative fold increase of OMVs determined using 3-deoxy-d-manno-octulosonic acid (Kdo). ****, $P < 0.0001$. (D) LOS levels associated with OMVs as indicated by silver staining following separation by SDS-PAGE.
Figure 3. Proteomic analysis of ST-OMVs.

Major protein functional categories (COGs) identified in *C. jejuni* 11168H ST-OMVs following proteomic analysis.

Figure 4. Increased proteolytic activity of ST-OMVs.

Quantification of proteolytic activity of OMVs isolated from 11168H cultured without ST or with 0.1% (w/v) or 0.2% (w/v) ST. FITC labelled casein was incubated with OMVs for 24 hr and OMV proteolytic activity assessed. **, *P* < 0.01; ****, *P* < 0.0001.

Figure 5. Increased cytotoxic activity and immunogenicity of ST-OMVs.

(A) Cytotoxic effect of OMVs isolated from 11168H in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST on T84 IECs after 24 hr co-incubation. The cytotoxic effect on the T84 cells was measured by quantifying the release of cytosolic lactate dehydrogenase (LDH) as a measure of cell damage. Non-challenged T84 cells represented 0% cytotoxicity (Uninfected Cells), and total lysis of T84 cells following treatment with 1% (v/v) Triton X-100 represented 100% cytotoxicity (Positive control). ***, *P* < 0.001. (B) Immunogenic effect of OMVs isolated from 11168H in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST on T84 IECs after 24 hr co-incubation. Levels of IL-8 secreted during *C. jejuni* OMV interactions with T84 cells were quantified using a human IL-8 ELISA. (C) Relative transcript levels of IL-8 in T84 cells co-incubated with OMVs from 11168H cultured without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Relative transcript levels of GAPDH was used as an internal control.
Figure 6. ST enhances the cytotoxicity of *C. jejuni* OMVs in the *Galleria mellonella* infection model.

*G. mellonella* larvae were injected with a 10 μl inoculum of OMVs (5 μg) from 11168H cultured in either Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Larvae were incubated at 37°C, with survival and appearance recorded every 24 h. PBS and no-injection controls were used. For each experiment, 10 *G. mellonella* larvae were infected and experiments were repeated in triplicate. *, *P* < 0.05.

Figure 7. qRT-PCR analysis of transcription in *C. jejuni* 11168H wild-type strain co-incubated with ST.

Quantitative real-time-PCR (qRT-PCR) analysis was performed using *cdtA, cdtB, cdtC, Cj0511, Cj1365c* and *htrA* transcript-specific primers. *gyrA* mRNA was used as an internal control. (A) The relative expression of *cdtA, cdtB, cdtC*. (B) The relative expression of *Cj0511, Cj1365c* and *htrA*. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Figure 8. Increased cleavage by ST-OMVs of Occludin and E-cadherin.

Recombinant Occludin or E-Cadherin were incubated with 10 μg of OMVs isolated from 11168H cultured in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Reaction mixtures were stopped and aliquots separated by SDS-PAGE and immunoblotted with antibodies against (A) Occludin or (B) E-Cadherin. Arrows indicate putative cleaved band. The negative control with phosphate buffered saline showed no detectable cleavage product.

Figure 9. Increased cleavage by ST-OMVs of GRP78/BiP.

(A) Recombinant GRP78 was incubated with 20 μg of OMVs isolated from 11168H cultured in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Reaction
mixtures were stopped, aliquots separated by SDS-PAGE and immunoblotted with a GRP78 antibody. Arrows indicate putative cleaved band. The negative control with phosphate buffered saline showed no detectable cleavage product. (B) Recombinant GRP78 was incubated with 20 μg of OMVs isolated from either htrA, Cj0511 or Cj1365c mutants cultured in Brucella broth and compared to 11168H OMVs as above.
REFERENCES


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